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Biosurfactant Production by *Streptomyces* sp. CGS B11 Using Molasses and Spent Yeast Medium

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Biosurfactants are considered a good alternative to highly-polluting petroleum-based surfactants that are toxic and non-biodegradable in nature. However, the high production cost of biosurfactants limits its potential for commercialization. The use of a highly efficient biosurfactant-producing actinomycetes isolate, combined with the utilization of low-cost substrates such as agro-industrial wastes, may aid in lowering the overall production cost. In this study, twenty-eight (28) actinomycetes isolated from distillery wastes and soil samples were screened for the production of extracellular biosurfactants. Based on the preliminary screening experiment, isolate CGS B11 – molecularly identified as *Streptomyces angustmyceticus* – produced the biosurfactant with the highest emulsification activity (E_{24}). Subsequently, the best alternative carbon and nitrogen sources, salt supplement, and pH level were determined using one-factor-at-a-time (OFAT) experiments. The highest measured biosurfactant activity was observed in the medium containing molasses, spent yeast autolysate, and NaCl, and a pH level ranging from 6.0–7.0. Biosurfactant production was observed to be growth associated with maximum emulsification activity achieved after 4 d of fermentation (late log phase). FTIR (Fourier transform infrared) spectra and biochemical composition analyses of the *S. angustmyceticus* CGS B11 biosurfactant suggest that it belongs to the lipopeptide type of biosurfactants. *S. angustmyceticus* CGS B11 biosurfactant also showed resistance and high stability on a wide range of temperature, pH, and salinity, and the ability to form stable and dense emulsions with various oils tested. It has great potential for various applications such as in the food and pharmaceutical industries, and in oil recovery and bioremediation. The results of this study will hopefully serve as a basis for large-scale production of biosurfactants utilizing agro-industrial wastes in the country.

Keywords: actinomycetes, biosurfactants, emulsification index, molasses, spent yeast, *Streptomyces* sp.

INTRODUCTION

Surfactants, also known as surface-active agents, are amphiphilic compounds that are capable of lowering surface tensions between two immiscible liquids (Piorr and Henkel 1987; Kawakatsu and Kawasaki 1990). Stable emulsions are primarily formed by the binding of hydrophobic compounds to the hydrophobic moiety of the biosurfactant molecules that in turn form micelles. With this mechanism, they may act as emulsifiers, detergents, foaming agents, dispersants, and wetting agents – depending on the purpose of their application. Hence, these compounds are widely utilized in so many

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industrial products such as personal care and cosmetic products, food products, cleaning agents, and for other several products for different applications (*e.g.* polymers, textile oils, paints) (Ying 2006; Schramm *et al.* 2003). Due to its many applications, the global surfactant market continues to grow at a rate of 6.02% from 2015–2019, with the rising demand for personal care products being at the forefront (Technavio 2015). However, surfactants are mainly derived from petroleum-based products, which are mostly toxic, non-renewable, and non-biodegradable.

One option to mitigate the deleterious environmental effects of petroleum-based surfactants is the production and use of biological-based surfactants, or biosurfactants, as alternatives. The mechanism of action of biosurfactants is similar to that of petroleum-based surfactants, but its use is more ecologically friendly. Unlike the chemically-synthesized surfactants, they have high biodegradability, low toxicity, multi-functionality, and environmental capability – making these compounds as promising alternatives for industrial and domestic applications (Akbari *et al*. 2018). Biosurfactants are known to have high activities at extreme levels of temperatures, pH, and salinity (Santos *et al.* 2016), and are capable of emulsifying even at very low concentrations because of its high kinetic stability (Uzoigwe *et al.* 2015; Schultz and Rosado 2020). Due to these advantages, biosurfactants have been used in oil bioremediation studies for formulating a safer, more effective, and stable remediation agent (Silva *et al.* 2014; Karlapudi *et al.* 2018; Effendi *et al.* 2018; Santos *et al.* 2017; Fenibo *et al.* 2019). There is also a rising application of biosurfactants in cosmetics, food-processing, and pharmaceutical industries where emulsions – specifically, nanoemulsions – are used to encapsulate, deliver, and protect food components such as oil-soluble flavors, vitamins, colorants, preservatives, and other bioactive ingredients (Aswathanarayan and Vittal 2019; Liu *et al.* 2019; Azmi *et al.* 2019).

The potential of biosurfactants also relies on the knowledge that it can be produced at a cheaper cost by utilizing inexpensive and renewable substrates (Banat *et al.* 2014; Olasanmi and Thring 2018). Agro-industrial wastes and crop residue are economical renewable substrates that are suitable for the production of biosurfactants, which have been reported by various related studies. Bran, beet molasses, rice straw, soy hulls, sugarcane molasses, whey, banana peels, corn cobs, and sugarcane bagasse are examples of agro-industrial by-products and crop residues that contain high amounts of residual sugars and lipids that can be used to support microbial growth in biosurfactant production (Banat *et al.* 2014; Rane *et al.* 2017; Tan and Li 2018). The use of these inexpensive substrates is important to reduce the production cost, which usually accounts for about 50% of the final product cost, and eventually make it more commercializable (Makkar and Cameotra 1997). A major consideration in biosurfactant production research is maximizing the product yield using microorganisms as an attempt to further decrease the total production cost.

Another important part of the strategy to enhance the potential of biosurfactants is an effective fermentative microorganism. Microorganisms such as yeasts, bacteria, and some filamentous fungi are capable of producing biosurfactants with different molecular structures and surface activities from agro-industrial wastes (Campos *et al*. 2013). Studies on biosurfactant production by commonly used bacteria, utilizing various agro-industrial wastes include *Pseudomonas aeruginosa* grown in blackstrap molasses (1.45 g/L yield) (Raza *et al.* 2007), *Streptococcus thermophilus* cultivated in molasses and cheese whey (1.4 g/L yield) (Rodrigues *et al.* 2006), and *Lactobacillus pentosus* grown in barley bran husk hydrolysate (0.28 g/L yield) (Moldes *et al.* 2007). Aside from these microorganisms, actinomycetes are also utilized for biosurfactant production.

Actinomycetes are Gram-positive filamentous bacteria that are abundant in soil, playing a major role in the recycling of material in nature (Shekhar *et al.* 2014). These organisms are non-pathogenic producers of bioactive metabolites with antimicrobial properties (Zambry *et al.* 2017). Due to this, they are considered as an appropriate candidate for safe microbial biosurfactant production. Biosurfactants from actinomycetes isolated from oil-contaminated soils and river sediments were shown to be promising for bioremediation (Panjiar 2013; Atuanya *et al.* 2016). However, their ability to produce biosurfactants are currently still less explored (Panjiar *et al.* 2013).

In this study, actinomycetes were isolated from distillery wastes and soil samples and were screened for the production of extracellular biosurfactant. The isolate producing the biosurfactant with the highest emulsification activity in the preliminary screening experiment was selected. Appropriate alternative carbon and nitrogen sources, salt supplement, and pH level were determined *via* OFAT experiments. The emulsification index (E_{24}) was monitored as the main response for determining the medium composition and fermentation condition that contributes positively to the production of the biosurfactant. The stability and activity of the biosurfactant towards various ranges of pH, temperature, and salinity were then investigated. Emulsification of the biosurfactant with various oils were also determined.

METHODOLOGY

Materials

Molasses and high fructose corn syrup (HFCS) were provided by Absolut Distillers, Inc. in Lian, Batangas, Philippines. Spent yeast and whey were obtained from Central Azucarera de Tarlac (CAT) in Tarlac City, Tarlac, and from the Dairy Training Research Institute, respectively.

Spent yeast autolysate was prepared following the protocol of Tanguler and Erten (2008). Briefly, 30 g of spent yeast was suspended in 200 mL of distilled water and the pH of the mixture was adjusted to 6.0. The suspension was incubated at 50 °C and 150 rpm for 16 h to efficiently extract the nitrogen from the cells. The mixture was centrifuged at 10,000 rpm for 15 min at 40 °C to separate the autolysate from cell debris. Finally, the autolysate was kept at 0 °C until use.

Actinomycetes were isolated from alcohol distillery wastes (soil compost, sludge, mudpress, coco dust, slops, wastewater, and effluent samples) from CAT in Tarlac City, Tarlac, and soil samples from selected areas in Albay (Cagsawa, Daraga, Malilipot, and Puro in Legazpi City).

Isolation, Characterization, and Preliminary Screening of Actinomycetes

Isolation and enumeration of actinomycetes were done by serial dilution and spread plating on starch casein agar (SCA) (soluble starch 1%, casein 0.03%, KNO_3 0.2%, $MgSO_4 \cdot 7H_2O$ 0.005%, NaCl 0.2%, $CaCO_3$ 0.002%, agar 1.8%). Plates were incubated at 30 °C for 7–10 d. Colonies with actinomycetes-like characteristics, being rough and elevated or embedded, were picked and streaked to new SCA plates. The isolates were also Gram-stained and observed under a compound light microscope (Olympus Biological microscope CX-40, Olympus Corporation, Tokyo, Japan) (10x magnification) to check for purity and ability to form mycelia.

A preliminary flask fermentation experiment was done to determine the best biosurfactant-producing actinomycetes isolate. Spore suspensions of the isolates with OD_{600} readings of 0.25 were prepared. One milliliter (1 mL) of the prepared spore suspension was then inoculated to 50 mL of glucose yeast extract (GYE) culture medium (peptone 1%, NaCl 0.5%, glucose 1%, yeast extract 0.3%) and incubated at ambient temperature with shaking at 150 rpm for 7 d. Supernatants containing the biosurfactant were collected by centrifugation, and emulsification activities were determined. The isolate producing the biosurfactant with the highest E_{24}, creating a stable or dense emulsion was considered for the OFAT experiments.

Molecular Identification Using 16s rDNA

The genomic DNA of isolate CGS B11 was extracted using Zymo Fungal / Bacterial DNA Mini Prep™ Extraction Kit by Zymo Research Corp., USA. PCR (polymerase chain reaction) amplification of the 16S rRNA gene sequence was done using primers 27f (AGAGTTTGATCMTGGCTCAG) and 1492r (TACGGYTACCTTGTTACGACTT). The PCR mixture contained 1x PCR buffer, 200 μM dNTPs, 1.5 mM $MgCl_2$, 20 μM of each primer, 1.25U *Taq* DNA polymerase, and 10 ng of the extracted DNA as the template. The following temperature profile was used for DNA amplification: initial denaturation at 95 °C for 5 min, followed by 30 cycles of denaturation at 95 °C for 1 min, annealing at 60 °C for 45 s, extension at 70 °C for 1 min, final extension at 70 °C for 10 min, and hold at 4 °C. PCR amplification was performed in T100™ Thermal Cycler (Bio-Rad, USA). The PCR product was run on a 1% (w/v) agarose gel electrophoresis containing GelRed™ (Biotium, USA), and was then viewed under ultraviolet light using the Syngene NuGenius Gel Documentation system to check product quality. The PCR product was subjected to capillary sequence analysis using the same primer pair on ABI 3730xl DNA Analyzer. Partial 16S rRNA sequence was compared to sequences available in the GenBank database using BLAST (Basic Local Alignment Search Tool; www.ncbi.nlm.nih.gov) to determine the possible identity of the isolate. Phylogenetic analysis was done by calculating genetic distances by the neighbor-joining (NJ) method using the *p*-distance method in MEGA X software. The result was used to build a dendrogram with 1000 bootstrap repetitions.

Determination of Carbon and Nitrogen Sources, pH, and Salt Supplement

Selected CGS B11 isolate was streaked on SCA plates and was allowed to grow for 5 d until aerial spores were observed. The plates were flooded with 10 mL sterile distilled water and the surfaces were gently scraped with sterile L-rod to dislodge the spores. The resulting spore suspension was transferred to a sterile screw-cap tube and subjected to vortex mixing for 2 min to eliminate the clumping of spores. The prepared spore suspension was then inoculated to the seed media (GYE) at a final optical density (OD) of 0.1. A mineral salt solution containing Na_2HPO_4 (2.2 g), KH_2PO_4 (1.4 g), $MgSO_4$ (0.6 g), $CaCl_2$ (0.04 g), and $FeSO_4$ (0.02 g) per L of distilled water was supplemented to the seed cultures to prevent flocculation, resulting to smaller clumps of cells. Inoculated seed media was incubated at ambient temperature for 24 h with shaking. This was then centrifuged at 10,000 rpm for 5 min to separate the seed broth culture. The OD of the resulting seed culture was measured at 600 nm using Shimadzu UV-1700 spectrophotometer and was inoculated in shake flask fermentation experiments with an initial OD_{600} of 0.5.

The determination of appropriate carbon and nitrogen

sources, as well as the pH and salt supplement, was done by OFAT experiments in a shake flask fermentation system for isolate CGS B11. One milliliter (1 mL) of the prepared spore suspension (OD_{600} of 0.25) of the isolate was inoculated to 50 mL of the fermentation medium. Flasks were then incubated at ambient room temperature with shaking at 150 rpm for 7 d.

The selection of alternative carbon (molasses and HFCS) and nitrogen (spent yeast autolysate and whey) sources was initially done. Concentrations of 1%, 2%, 3%, 4%, and 5% of these alternative carbon and nitrogen sources were substituted to the glucose and yeast extract components, respectively, of the GYE medium. The combination of carbon and nitrogen sources (together with their corresponding concentrations) producing the biosurfactant with the highest emulsification activity was selected.

Subsequently, the appropriate salt supplement and pH for biosurfactant production were determined in a separate similar experiment. Different concentrations (0.1%, 0.2%, 0.4%, 0.5%, 0.6%, 0.8%, and 1.0%) of various salts (NaCl, $MgCl_2$, and $CaCl_2$) were considered, and different pH levels (5.0, 6.0, 7.0, and 8.0) were also tested. Biosurfactant production by CGS B11 was done in shake flasks at 30 °C for 7 d. Similarly, the combination of pH and salt supplement (together with the corresponding concentration) producing the biosurfactant with the highest emulsification activity was selected.

Testing of Biosurfactant Stability

Effects of temperature, pH, and salinity to the emulsification activity of the biosurfactant produced by CGS B11 were determined by subjecting the biosurfactant produced to different temperatures (4, 10, 25, 30, 40, 50, 60, 70, 80, and 100 °C) for 15 min, adjusting its pH (5, 6, 7, and 8), or by adding NaCl (1%, 2%, 3%, 4%, 5%, 6%, 7%, and 8%) (Khopade *et al.* 2012). Also, to determine the capability of the biosurfactant to produce stable emulsions with different hydrophobic substances, different plant-based oils (canola, corn, coconut, palm, olive, soy, sunflower, and used vegetable oil) were tested for emulsification activity.

Analytical Methods

Biomass concentration by dry cell weight. Fermentation broth samples (1 mL per sample) were centrifuged at 10,000 rpm for 10 min to separate the cells from the supernatant. The solids were washed with distilled water and dried in an oven at 105 °C for 24 h. Weight was monitored until constant.

Emulsification activity. Emulsification activity was measured by determining the emulsification index (E_{24}) of the biosurfactant (Nayarisseri 2018). Briefly, the culture broth containing the biosurfactant was separated from bacterial cells by centrifugation at 4,000 rpm for 10 min. Three milliliters (3 mL) of the cell-free broth culture was mixed with 3 mL of kerosene (55.2% paraffins, 40.9% naphthenes, and 3.9% aromatics) (US EPA 2011) in a dram vial using a high-speed vortex mixer for 2 min (Sarrubo *et al.* 2001) (note: kerosene was replaced with various hydrophobic substances during the testing of biosurfactant for emulsification towards different oils). Mixtures were allowed to rest for 24 h and the resulting emulsion layers were measured using a digital caliper. The emulsification index was then calculated using the formula:

$$E_{24} = \frac{\text{height of emulsion layer}}{\text{total mixture height}} \times 100$$

Characterization of the Biosurfactant

The protein composition of the biosurfactant was determined by spectrophotometry using Shimadzu UV-1700 spectrophotometer (Lowry *et al*. 1951). Additionally, the lipid composition of the biosurfactant was analyzed by Folch organic extraction (Wenk 2016). About 50 mL of the cell-free culture broth was mixed with 50 mL chloroform-methanol (2:1 v/v) with continuous vigorous shaking for 30 min in a separatory funnel. The organic layer and aqueous layers were allowed to separate. The organic layer containing the lipids was air-dried until a constant weight was achieved. The mass of the extracted lipid was then determined.

Thirty milliliters (30 mL) of the frozen crude biosurfactant of CGS B11 was freeze-dried. Exactly 0.1 g of the freeze-dried biosurfactant was used for FTIR analysis using Shimadzu Spectrometer Model AS220/C/2, Japan. Infrared spectra of the sample were collected over the range of 800–4000 cm^{-1}.

RESULTS AND DISCUSSION

Isolation, Characterization, and Preliminary Screening

A total of 28 actinomycetes were isolated from various distillery and soil samples. Table 1 shows the cultural characteristics and gram stain reactions of the purified actinomycetes isolates grown in SCA plates. All isolates had the rough surface texture of colonies, either flat or embedded in the agar, and the presence of aerial spores that were of black or white in color (Figure 1). Microscopic examination of the isolates revealed that most of the isolates had an extensive formation of mycelia and were Gram-positive. A notable earthy smell was observed on all isolates, which is due to the geosmin, a neutral oil substance being

Table 1. Colony characteristics and Gram reaction of isolated actinomycetes grown on SCA.

Organism (code name)	Growth on SCA		
	Colonies	Aerial spores (fuzz)	Gram reaction
CADT[1] 1	Rust, flat	White	Negative
CADT[1] 2	Cream, flat	White	Positive
CADT[1] 3	Pale yellow, flat	White	Positive
CADT[1] 4	Yellow, flat	Absent, shiny	Positive
CADT[1] 5	Cream, flat	White	Positive
CADT[1] 6	Brown, flat	White	Negative
CADT[1] 7	Brown, umbonate	White	Positive
CADT[1] 8	Cream, umbonate	White	Negative
CADT[1] 9	Cream, flat	White	Positive
CADT[1] 10	Brown, umbonate	White	Positive
CADT[1] 11	Brown, flat	White	Positive
CADT[1] 12	Cream, umbonate	White	Positive
APJ[2] 1	Cream, flat	Absent, shiny	Positive
APJ[2] 2	Yellow, flat	White	Positive
APJ[2] 3	Brown, raised	White	Positive
APJ[2] 4	Tan, umbonate	White	Positive
APJ[2] 5	Cream, umbonate	White	Positive
VAA[3] 1	Red, umbonate	White	Positive
VAA[3] 2	Orange, umbonate	White	Positive
JSM[4] 8	Brown, flat	Absent, shiny	Positive
CGS[5] A9	Brown, crateriform	White marginal	Positive
CGS[5] B11	Cream, convex	White	Positive
BDY[6] A4	Beige, umbonate	Absent	Positive
CGS[5] A10	Yellow, raised	White	Positive
UNO[7] B2	Yellow, crateriform	White	Positive
BDY[8] B13	Moss green, flat	White to black	Positive
PURO[9] B2	Yellow, umbonate	White	Positive
UNO[7] C14	Light Brown, umbonate	Absent	Positive

CADT[1], APJ[2], VAA[3], JSM[4], BDY[6] – samples from CAT, Tarlac City
CGS[5] – soil from Cagsawa, Albay
PURO[9] – soil sample from Puro, Legazpi City, Albay
UNO[7] – soil sample from Barangay Uno, Malilipot, Albay
BDY[8] – soil sample from Barangay Budyao, Daraga, Albay

produced by actinomycetes as one of its metabolites (Lee *et al.* 2011). Both terrestrial and marine environments serve as habitats for actinomycetes, where soil remains the most significant source (Anandan *et al.* 2016).

After the cultural and morphological characterization of the isolates, flask fermentation experiments were done to determine the best biosurfactant-producing actinomycetes isolate. Table 2 shows the E_{24} values of the tested isolates, where a minimum value of 12.00% and a maximum value of 47.14% were obtained from isolates APJ5 and CADT 7, respectively. Initially, the emulsification indices of the isolates were used as the basis to choose the best isolate for the OFAT study. However, the consistency of emulsions – whether loose, compact, or dense – was also considered (Figure 2). Biosurfactants with the capability to make dense emulsions had the better stabilizing capacity, creating more stable emulsions. Effective biosurfactants inhibit the aggregation of droplets by forming a protective coating around the droplets and, thus, making stable emulsions (Ralla *et al.* 2017). Hence, isolates with dense emulsions were considered over the isolates that had compact and loose emulsions.

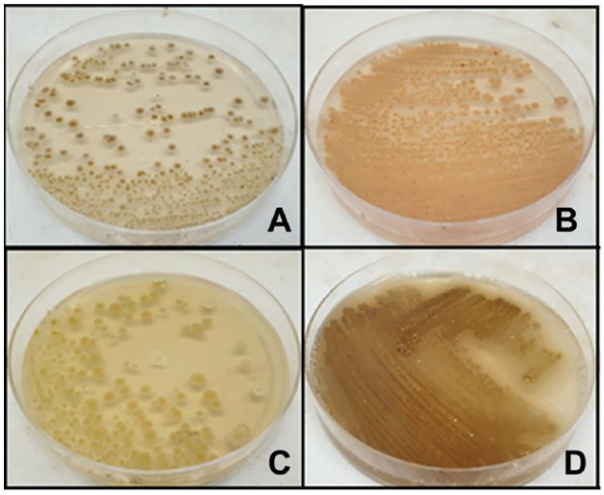

Figure 1. Some of the isolated actinomycetes from distillery wastes and soil samples: (A) CGS B11, (B) *Streptomyces* sp., (C) Puro B2, and (D) JSM 8.

Isolate CGS B11 produced a biosurfactant that had an E_{24} value of 43.58% and an intensely dense emulsion consistency and, hence, was chosen for the succeeding experiments. Moreover, the isolate had comparatively the fastest observable growth, being able to grow and produce a biosurfactant with high emulsification activity in the GYE medium after 48 h. Although some isolates had higher E_{24} values, such as CGS A10 (46.43%) and BDY B13 (45.71%), CGS B11 was still chosen because of its faster growth and ability to form finer aggregates of cells, which was ideal for standardizing the inoculum size in the fermentation experiments. Interestingly, all the emulsions formed by the biosurfactants from all the isolates were stable at room temperature despite the differences in emulsification activities, indicating their potential as bioemulsifiers.

Table 2. Emulsification activities (E_{24}) and emulsion consistencies of the isolated actinomycetes.

Organism (code name)	Emulsification index (E_{24}), %	Emulsion consistency
CADT 1	45.71	Intense
CADT 2	42.86	Intense
CADT 3	0.00*	None
CADT 4	36.86	Intense
CADT 5	20.86	Intense
CADT 6	36.86	Intense
CADT 7	47.14	Intense
CADT 8	42.86	Intense
CADT 9	25.71	Loose
CADT 10	40.86	Intense
CADT 11	28.57	Loose
CADT 12	45.71	Intact
APJ 1	42.86	Intense
APJ 2	34.29	Intact
APJ 3	22.86	Loose
APJ 4	42.86	Intact
APJ 5	12.00	Loose
VAA 1	17.14	Loose
VAA 2	20.00	Loose
JSM 8	0.00*	None
CGS A9	15.72	Loose
CGS B11	43.58	Intense
BDY A4	37.15	Intact
CGS A10	46.43	Intense
UNO B2	25.71	Intact
BDY B13	45.71	Intense
PURO B2	21.43	Intact
UNO C14	0.00*	None

*No emulsification activity

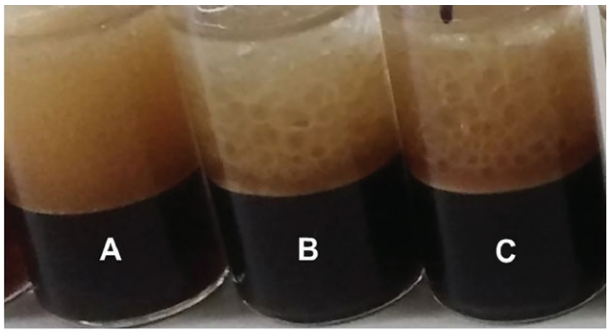

Figure 2. Consistency of emulsion layers: (A) dense, (B) loose, and (C) compact.

Identity of Putative Actinomycete Isolate

Analysis of the 16S rRNA sequence was carried out to molecularly identify isolate CGS B11. The PCR amplified product of the partial 16S rRNA gene of the isolate was sequenced with a total length of 1269 bp. A BLAST analysis carried out through blast search through GenBank (http://www.ncbi.nlm.nih.gov) revealed that the species most closely related to the CGS B11 was *Streptomyces angustmyceticus*, with an overall identity score of 100% and with no mismatches. The reference was from a recently described species, *S. angustmyceticus* (strain NBRC 3934), within the *Streptomycetales* order, *Streptomycetaceae* suborder, and *Streptomyces* genus, with no known source of isolation (not indicated on the database). The isolate also showed good identity (99%) with other members of the genus *Streptomyces* within the GenBank including *S. nigrescens*, *S. libani*, *S. tubercidicus*, *S. caniferus*, *S. catenulae*, *S. glebosus*, *S. sioyaensis*, *S. platensis*, *S. ramulosus*, *S. decoyicus*, *S. ossamyceticus*, and *S. chattanoogensis*. A non-*Streptomyces* genus was also closely related to the inquiry with 99% similarity, the *Kitasatospora misakiensis* strain IFO 12891, which is under the same order and suborder of the genus *Streptomyces*.

Phylogenetic analysis of CGS B11 (Figure 3) was then constructed based on the NJ tree using its 16S rRNA sequence, which was compared with other species of *Streptomyces sp*. with high sequence similarity from the NCBI database. A bootstrap score of 96 was observed between isolate CGS B11 and the other two *S. angustmyceticus* strains, while other *Streptomyces* species were related but had much lower bootstrap scores.

Determination of Carbon and Nitrogen Sources, pH, and Salt Supplement

Molasses as a carbon source at 1% (w/v) concentration was found to be suitable for *S. angustmyceticus* CGS B11 biosurfactant production, giving an E_{24} value of 74.66%. This was comparable to the 72% E_{24} value of *S. angustmyceticus* CGS B11 biosurfactant produced in a medium containing 1% (w/v) of HFCS as a carbon source (Figure 4). The increase in molasses concentration up to 6% (w/v) did not have a significant effect on the E_{24} of the produced biosurfactant. Due to the viscosity of the medium, further increasing the amount of molasses in the medium may have inhibited the growth of the isolate, making 1% (w/v) of molasses the ideal concentration for biosurfactant production. The lower requirement of molasses concentration to produce a biosurfactant with high emulsification activity contributes to the potential economical production of the biosurfactant by the isolate. It could be explained by the presence of compounds other than sugar in the molasses, like proteins

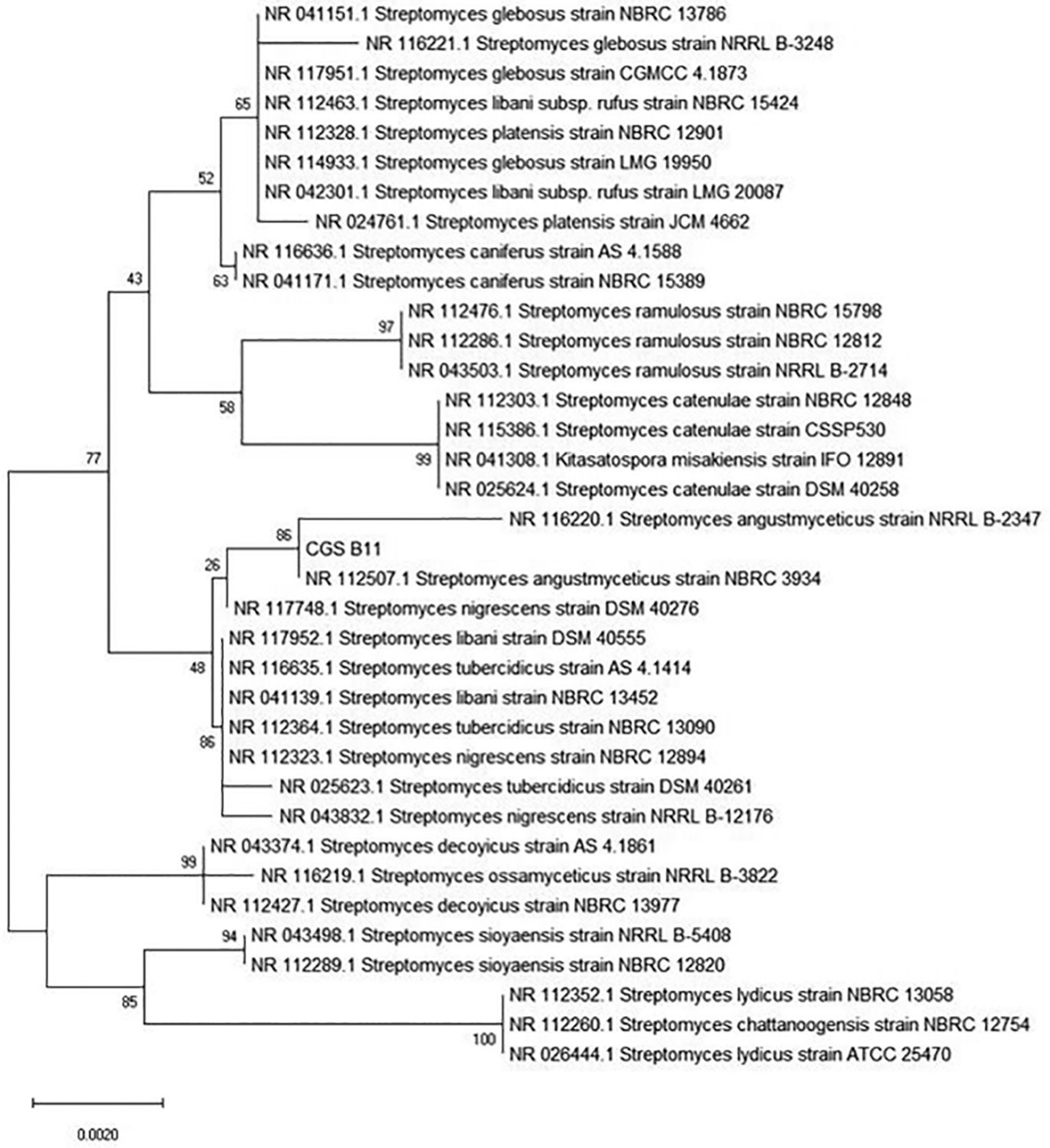

Figure 3. Phylogenetic relationship of isolate CGS-B11 with other *Streptomyces* strains based on partial 16S rRNA gene sequences.

and minerals, which may have contributed to the increased production of biosurfactant (Mouafo *et al.* 2018). The pairwise comparison of E_{24} values at different molasses concentrations can be found in the supplementary material (Appendix I).

Sugarcane molasses is a by-product in the sugarcane industry, which is the final effluent in the process of sugar refinery. It is a low-cost substrate compared to HFCS and other conventional sugar sources like sucrose and glucose (Tan and Li 2018). It also has high total sugar content – mainly sucrose – at a level of about 40% (w/w), and contents of other compounds such as minerals, organic compounds, and vitamins (Saimmai *et al.* 2011). Sugarcane molasses has been used for decades for the production of various industrially important bioproducts, including biosurfactants. It is evidenced by many studies as a good

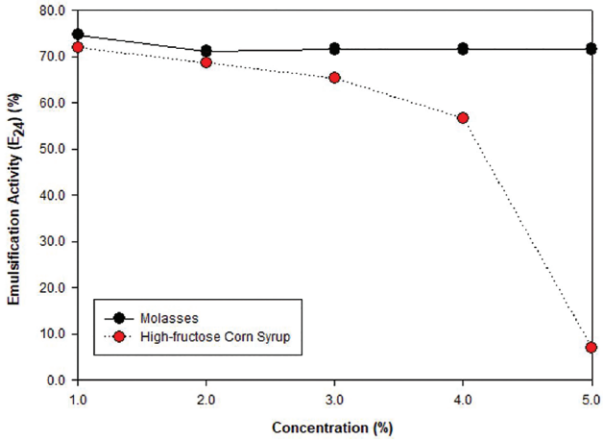

Figure 4. Emulsification activity (E_{24}) of the biosurfactant produced by isolate CGS B11 grown at different concentrations of molasses and HFCS.

low-cost substrate for enhancing biosurfactant production of various bacterial strains for various applications. In a study by Suryanti *et al.* (2015), sugarcane molasses was found best for the production of rhamnolipids, which is a type of biosurfactant, of *Pseudomonas fluorescens*. The use of sugarcane molasses as a substrate improved the antimicrobial biosurfactant production of *Lactobacillus paracasei* subsp. *tolerans* N2 (Mouafo *et al.* 2018). Other bacteria, such as *Azotobacter vinelandii*, was reported to utilize sugarcane molasses with high potential for biosurfactant production (Devianto *et al.* 2020). In a similar study, using molasses as a carbon source yielded more biosurfactant by *Lactobacilli* strains compared to that of glycerol and the standard medium de Man Rogosa and Sharpe (MRS) broth (Mouafo *et al.* 2018).

On the other hand, as shown in Figure 5, spent yeast autolysate was selected as the most appropriate nitrogen source. At 4% (v/v) concentration, a biosurfactant with an E_{24} value of 56% was produced. This value was significantly higher compared to that of whey (37%) at 6% concentration. Spent yeast autolysate concentration above or below 4% significantly lowered the emulsification activity, implying that an adequate amount of nitrogen that is not too high or not too low is needed by the isolate to synthesize the biosurfactant efficiently. The pairwise comparison of E_{24} values at different spent yeast autolysate concentrations can be found in the supplementary material (Appendix II). Spent yeast is a by-product in alcohol production and is considered as a good nitrogen source, containing about 3.9% total nitrogen on a dry solid basis and other valuable and bioactive substances (Tanguler and Erten 2008). Yeast extract and -glucans are the main products that are currently being obtained from spent yeast as it also contains other valuable and bioactive substances (Rakowska *et al.* 2017). A study by Alcantara *et al.* (2012) showed the potential of utilizing spent yeast for more economical production of biosurfactant by *Saccharomyces cerevisiae* 2031. Other than biosurfactant production, spent yeast is also used in succinic acid production and ethanol fermentation from sweet sorghum juice, replacing yeast extract as a nitrogen source (Jiang *et al.* 2009; Sridee *et al.* 2011). This study is the first to report the use of spent yeast autolysate for the enhancement of the production of biosurfactant by actinomycetes.

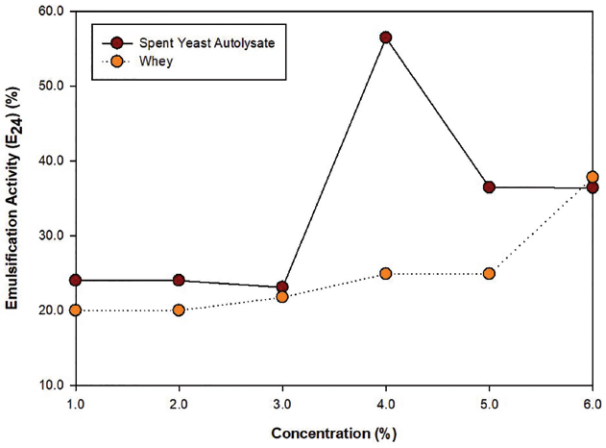

Figure 5. Emulsification activity (E_{24}) of the biosurfactant produced by isolate CGS B11 grown at different concentrations of spent yeast autolysate and whey.

The utilization of molasses and spent yeast as alternative substrates for biosurfactant production would help lessen pollution and expenses in the waste disposal and waste treatment in sugar industries and alcohol distilleries. These major agro-industrial by-products often cause major problems when disposed of improperly, leading to organic material contamination of natural water sources (Zechner-Krpan *et al.* 2010).

After determining the appropriate carbon and nitrogen sources, salt supplement, and pH level requirements for biosurfactant production by *S. angustmyceticus* CGS B11 were investigated. The addition of 0.5% NaCl in the production medium gave the highest E_{24} value of 52%, followed by $MgCl_2$ (44%), and $CaCl_2$ (32%). The pairwise comparison of E_{24} values using these salts can be found in the supplementary material (Appendix III). This agrees with the results of a related study by Maniyar *et al.* (2011), where 0.5% NaCl was found optimum for the maximum bioemulsifier production by *Rhodococcus* sp. Additionally, biosurfactant production by *Acinetobacter junii* SC14 was inhibited by $MgCl_2$ and $CaCl_2$. The salt concentration of a particular medium has a corresponding effect on the biosurfactant production of a microorganism as their cellular activities are affected by salt concentration (Fakruddin 2012). In addition, the increase in the concentration of salts causes the destabilization of emulsions due to the disturbances in

electrostatic forces between droplets (Rocha e Silva *et al.* 2017). Some biosurfactants, however, are not affected by salt concentrations of up to 10% (w/v) with only slight reductions in activity (Abu-Ruwaida *et al.* 1991).

Meanwhile, a good pH level for biosurfactant production of *S. angustmyceticus* CGS B11 was found to be at pH 6.0–7.0, resulting in an average biosurfactant emulsification activity of 75% (Figure 6). The pairwise comparison of E_{24} values at different initial pH values can be found in the supplementary material (Appendix IV). Adverse effects on E_{24} were observed at more acidic and basic pH levels. Lower emulsification indices of 62.09% and 52.89% were obtained at pH 5.0 and pH 8.0, respectively. In a similar study, maximum bioemulsifer production by *Streptomyces* sp. S22 was at pH 6.0 and least at pH 5.0 (Maniyar *et al.* 2011). Kokare *et al.* (2007) also reported that *Streptomyces* sp. S1 showed maximum biosurfactant production at neutral pH. As Manivasagan *et al*. (2014) mentioned, the pH of the medium is vital for the cell growth of microorganisms and the production of secondary metabolites. It is also important to mention that each microorganism adapts to a specific pH for each type of biosurfactant to be produced (Rufino *et al.* 2008) Extreme pH affects the structure of biomolecules, where hydrogen bonds between strands of DNA breaks at high pH, while lipids are hydrolyzed by extremely basic pH (Turner 2018).

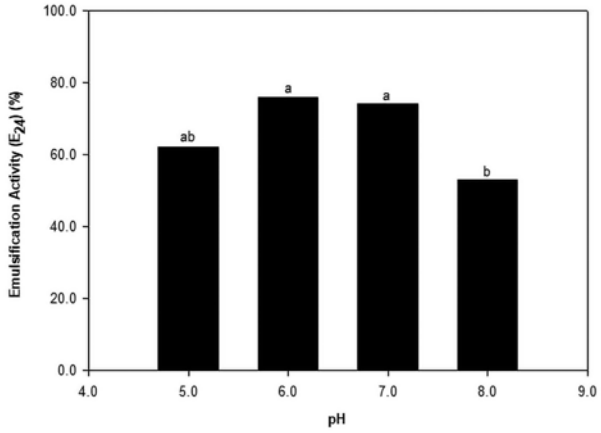

Figure 6. Emulsification activity (E_{24}) of the biosurfactant produced by isolate CGS B11 in medium at varying pH levels. Effect of treatment is significant as determined by one-way ANOVA ($p \leq 0.01$). Values with a common letter are not significantly different using Tukey's test at 5% level.

Fermentation Kinetics of Biosurfactant Production

Figure 7 shows the growth-associated production of biosurfactant by *S. angustmyceticus* CGS B11 using the improved medium for biosurfactant production. The stationary phase was observed after 24 h of fermentation time, where the produced biosurfactant, had an E_{24} value of 20.00%. The E_{24} value drastically increased to 56% after 48 h of fermentation. Maximum biosurfactant emulsification activity of 66.96% was achieved after 5 d of fermentation and remained significantly constant after 6 d (68.00%) and 7 d (67.66%). This implies that biosurfactant production occurs during the late log phase up to the stationary phase of the microbial growth. A similar observation was made by Sarubbo *et al*. (2001) wherein two strains of yeast *C. lipolytica* were found to produce biosurfactant during the late log phase to the early stationary phase. On the other hand, in a similar study, maximum biosurfactant production (2.24 g/L) and emulsification activity by *Streptomyces* sp. DPUA1559 was observed during the stationary phase after 4.5 d (Santos *et al.* 2018).

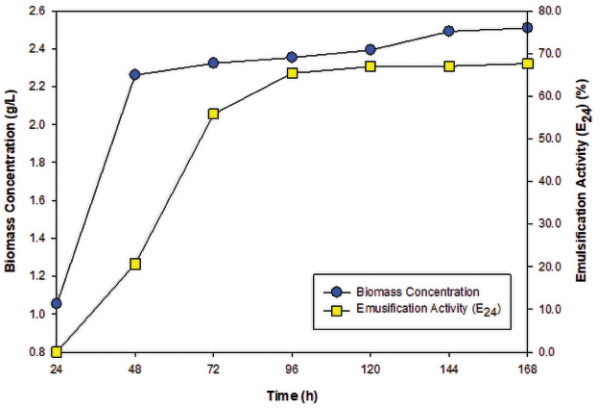

Figure 7. Fermentation kinetics of biosurfactant production by isolate CGS B11 using the improved medium for biosurfactant production.

Stability Testing of Biosurfactant

Results showed that the biosurfactant produced by *S. angustmyceticus* CGS B11 grown in the formulated medium has stable emulsification activity at different levels of temperatures, salinity, and pH – with average E_{24} values of 71.3%, 68.1%, and 70.9%, respectively. This agrees with the results of a related study by Santos *et al.* (2018), wherein the biosurfactant from *Streptomyces* sp. DPUA 1559 exhibited thermal and pH stability and tolerance under high salinity. Another study on *Streptomyces* sp. biosurfactant showed high stability on a wide range of temperature, pH, and salt concentration (Atuanya *et al.* 2016). Stability of the biosurfactant at different temperatures makes it useful in food, pharmaceuticals, and cosmetics industries since sterilization at high temperatures and storage at low temperatures are common processes involved in the production (Elazzazy *et al.* 2015). Furthermore, the stability of the biosurfactant at different pH and salinity shows its applicability in soap production, acidic food products, and bioremediation (Prieto *et al.* 2008; Phulpoto *et al.* 2020; Araújo *et al.* 2019).

The biosurfactant from isolate *S. angustmyceticus* CGS B11 also formed stable and dense emulsions with all the oils tested. However, E_{24} values obtained were significantly different, which could be due to the differences in the fatty acid components of the oils (Alcantara *et al.* 2010). Coconut (100%), soy (88.4%), and used oils (85.8%) were highly emulsified while olive (47.3%), sunflower (45.6%), and canola (42.8%) oils were only moderately emulsified. Likewise, rhamnolipids from *Pseudomonas aeruginosa* RB 28 emulsified various types of oils – including vegetable, corn, olive, and sunflower, forming stable emulsions (Sifour *et al.* 2007). These properties hint the potential of biosurfactants for bioremediation of oil-contaminated sites and oil recovery.

Characterization of Biosurfactant

The chemical nature of the freeze-dried biosurfactant sample obtained from isolate *S. angustmyceticus* CGS B11 using the improved medium was determined by the FTIR spectra analysis, as shown in Figure 8. Peaks recorded at 3352.28 cm^{-1} and 1629.85 cm^{-1} were corresponding to the N-H stretching vibrations (a typical characteristic of aliphatic primary amine group) and the presence of C=C stretching belonging to an alkane group. Additionally, the peak at 1552.70 cm^{-1} is due to the N-O stretching of a nitro compound, while the peak at 1450.47 cm^{-1} represents the C-H bending of a methyl group. Lastly, peaks at 1400.32 cm^{-1}, 1246.02 cm^{-1}, 1157.29 cm^{-1}, and 1076.28 cm^{-1} are due to carboxylic acid, amine, tertiary, and primary alcohol groups, respectively. These results strongly indicate that the biosurfactant contained aliphatic and peptide-like moieties, thus confirming the lipopeptide nature of the crude biosurfactant (Thaniyavarn *et al.* 2014; Biniarz *et al.* 2015). Lipopeptide biosurfactant was also identified in other studies using actinomycetes. Various related studies on actinomycetes, including isolates from the marine environment, revealed the production of lipopeptide biosurfactant, which could be developed for large-scale production (Kiran *et al.* 2017; Zambry *et al.* 2017).

CONCLUSIONS

This study showed that actinomycetes isolated from various sources were able to produce extracellular biosurfactant when cultured in the GYE medium. From these isolates, isolate CGS B11 – characterized as *S. angustmyceticus* based on morphological and molecular characterization – was selected as the main biosurfactant producer for further studies on improving the fermentation process using various waste substrates and fermentation conditions. Results of the OFAT experiments revealed that molasses and spent yeast autolysate were the best carbon and nitrogen sources at 1% (w/v) and 4% (v/v), respectively, for the production of *S. angustmyceticus* CGS B11 biosurfactant. Additionally, NaCl at 0.5% (w/v) concentration and pH range of 6.0–7.0 was found to enhance the biosurfactant production of the isolate. The partial characterization done on the biosurfactant produced by *S. angustmyceticus* CGS B11 suggests that it belongs

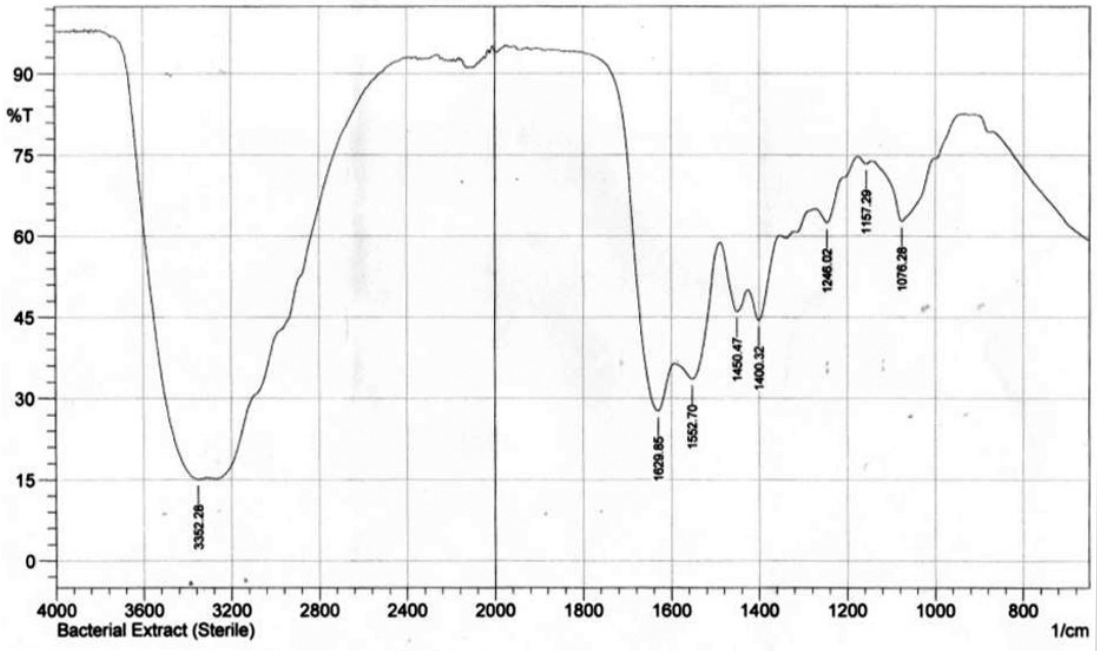

Figure 8. FTIR spectrum of freeze-dried biosurfactant from isolate CGS B11 grown in the improved molasses and spent yeast autolysate medium.

to the lipopeptide type of biosurfactants. Remarkably, this biomolecule has promising characteristics – showing resistance and high stability across a wide range of pH, temperature, and salinity – indicating its potential for a wide range of applications in various food and pharmaceutical industries. Moreover, emulsions of the biosurfactant with various oils tested were stable and dense, suggesting its great potential for applications in oil recovery and bioremediation applications. Thus, utilizing *S. angustmyceticus* CGS B11 and employment of wastes from sugar refining and alcohol industries presents a way in developing a strategy for more economic production of this promising biomolecule.

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NOTE ON APPENDICES

The complete appendices section of the study is accessible at http://philjournsci.dost.gov.ph

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APPENDICES

Appendix I. Pairwise comparison of emulsification activities (E24) at different molasses concentrations. Values with different superscripts are significantly different.

Molasses concentration (w/v)	Emulsification index (E_{24}), %
1%	74.66 ± 2.31^{a}
2%	71.11 ± 1.54^{ab}
3%	71.56 ± 0.77^{ab}
4%	71.56 ± 1.53^{b}
5%	71.56 ± 0.78^{c}

Appendix II. Pairwise comparison of emulsification activities (E24) at different spent yeast autolysate concentrations. Values with ifferent superscripts are significantly different.

Spent yeast autolysate concentration (w/v)	Emulsification index (E_{24}), %
1%	36.00 ± 1.88^{a}
2%	34.67 ± 28.28^{ab}
3%	64.66 ± 0.94^{ab}
4%	53.33 ± 15.08^{b}
5%	23.33 ± 27.34^{c}

Appendix III. Pairwise comparison of emulsification activities (E24) using different salts. Values with different superscripts are significantly different.

Salt	Emulsification index (E_{24}), %
Sodium chloride	51.55 ± 8.57^{a}
Magnesium chloride	43.55 ± 2.04^{ab}
Calcium chloride	32.45 ± 6.84^{b}

Appendix IV. Pairwise comparison of emulsification activities (E24) at different pH values. Values with different superscripts are significantly different.

pH	Emulsification index (E_{24}), %
5.0	62.09 ± 8.04^{ab}
6.0	76.00 ± 2.67^{a}
7.0	74.22 ± 4.29^{a}
8.0	52.89 ± 11.65^{b}